Triadimefon, a fungicidal triazole-type P450 inhibitor, induces brassinosteroid deficiency-like phenotypes in plants and binds to DWF4 protein in the brassinosteroid biosynthesis pathway

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Triadimefon (Bayleton®), a widely used triazole-type fungicide, affects gibberellin (GA) biosynthesis and 14α-demethylase in sterol biosynthesis. The present study revealed that the phenotype of *Arabidopsis* treated with triadimefon resembled that of a brassinosteroid (BR)-biosynthesis mutant, and that the phenotype was rescued by brassinolide (BL), the most active BR, partly rescued by GA, and fully rescued by the co-application of BL and GA, suggesting that triadimefon affects both BR and GA biosynthesis. The target sites of triadimefon were investigated using a rescue experiment, feeding triadimefon-treated *Arabidopsis* BR-biosynthesis intermediates, and a binding assay to expressed DWF4 protein, which is reported to be involved in the BR-biosynthesis pathway. The binding assay indicated that the dissociation constant for triadimefon was in good agreement with the activity in an *in planta* assay. In the triadimefon-treated *Arabidopsis* cells, the *CPD* gene in the BR-biosynthesis pathway was up-regulated, probably due to feedback regulation caused by BR deficiency. These results strongly suggest that triadimefon inhibits the reaction catalysed by DWF4 protein and induces BR deficiency in plants. As triadimefon treatment has proved to be beneficial to plants, this result suggests that BR-biosynthesis inhibitors can be applied to crops.

Key words: ergosterol biosynthesis, gibberellin, plant growth regulator.

INTRODUCTION

Plant cytochrome P450 mono-oxygenases participate in many biochemical pathways, including some essential P450 functions that are conserved among plant species, such as those involving hormones, sterols and oxygenated fatty acids [1]. Therefore regulation of cytochrome P450 mono-oxygenases by the mutation of genes encoding these enzymes or specific inhibitors targeting these enzymes should result in serious defects in plant development. Brassinosteroids (BRs) are important plant hormones [2,3], and the involvement of P450s in their biosynthetic pathway was indicated by the identification of *CPD* [4] and *DWF4* [5] from *Arabidopsis* mutants and *DWARF* [6] from tomato mutant and *DDWF1* [7] in pea. The *Arabidopsis* mutants resemble light-grown plants when grown under etiolated conditions. The *dwf4* and *cpd* mutants revealed that the *DWF4* and *CPD* loci each encode a cytochrome P450 mono-oxygenase. The C-22 and C-23 positions of BRs are thought to be successively hydroxylated by DWF4 and CPD respectively.

We have reported previously that two BR-biosynthesis inhibitors, brassinazole [8–10] and Brz2001 [11], and their pyrimidine derivatives [12] induce dwarfism in *Arabidopsis*; the resulting plant resembles a BR-biosynthesis mutant and can be rescued by BRs. These BR-biosynthesis inhibitors proved to be a useful tool to investigate the role of BRs in plants and to find mutants in which genes involved in plant-hormone signal transduction were altered [13,14]. Since brassinazole and Brz2001 (Figure 1) belong to the chemical class of triazole derivatives that act by inhibiting cytochrome P450s, it is reasonable to assume that these inhibitors also block the steps catalysed by cytochrome P450. In fact, direct analysis of the interaction between DWF4 protein expressed in *Escherichia coli* and brassinazole and its derivatives has revealed that brassinazole targets C-22 hydroxylation catalysed by DWF4 to induce BR deficiency in plants [15].

Triadimefon (Bayleton®), a widely used fungicidal triazole-type P450 inhibitor shown in Figure 1, not only interferes with oxidative demethylation reactions in the ergosterol-biosynthesis pathway of fungi, but also blocks gibberellin (GA) biosynthesis [16]. In addition to GA-biosynthesis inhibition, triadimefon induces several other plant growth responses: treated plants accumulate zeatin and have cytokinin-like activity with anti-senescence properties, and they are also shorter and more compact, with thicker and darker green leaves [17]. These side effects of triadimefon have proved beneficial to plants [18,19]. We noticed that the phenotypes induced by triadimefon are like those of BR-deficient plants [20,21], and differ from those induced by GA-biosynthesis inhibitors. In this context, we investigated

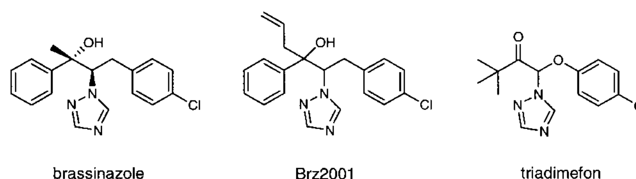

Figure 1 Chemical structures of brassinazole and triadimefon

Abbreviations used: BL, brassinolide; BR, brassinosteroid; GA, gibberellin; RT, reverse transcriptase.

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the potency of triadimefon in inhibiting BR biosynthesis in the expectation of developing BR-biosynthesis inhibitors as new plant-growth regulators. The assessment involved comparing the phenotypes induced by triadimefon with those of the BR-deficient mutant *det2* by feeding plants BR-biosynthesis intermediates and measuring the recovery from inhibitor treatment, and by measuring the dissociation constants of the DWF4 protein expressed in *E. coli* for triadimefon. Triadimenol, in which a carbonyl group of triadimefon is reduced to a hydroxy group, showed only weak inhibitory activity against BR biosynthesis.

MATERIALS AND METHODS

Chemicals

The brassinazole used in this study was synthesized and purified as reported previously [22]. Triadimefon and triadimenol were purchased from Wako Pure Chemical Industries, Ltd (Tokyo, Japan). Brassinolide (BL) and castasterone were purchased from CIDtech Research Inc. (Cambridge, ON, Canada). The other intermediates in the BR-biosynthesis pathway used in this report were synthesized as described previously [23–25].

Plant materials and growth conditions

Seeds of *Arabidopsis* (ecotype Columbia) were purchased from LEHLE Seeds (Round Rock, TX, U.S.A.). *Arabidopsis det2* seeds were a gift from Dr J. Chory (Salk Institute, CA, U.S.A.). Cress (*Lepidium sativum*) seeds were purchased locally. *Arabidopsis* seeds were cold-treated (4 °C) for 2 days, and then surface-sterilized in 1 % (v/v) NaOCl for 20 min before washing with sterile distilled water five times. Seeds were sown on 1 % agar-solidified medium containing half-strength Murashige and Skoog salts and 1.5 % (w/v) sucrose in plastic plates with or without chemicals. The plates were sealed with Parafilm (American National Can Co., Chicago, IL, U.S.A.) and the plants were grown in the dark in a growth chamber for 10 days at 22 °C. Cress seeds were sown in 0.8 % agar-solidified medium containing half-strength Murashige and Skoog salts and 1.5 % (w/v) sucrose in Agripots (Kirin Brewery Co., Tokyo, Japan) with or without chemicals. Cress seeds were grown in a 16 h:8 h light (120 μmol · $m^{-2} \cdot s^{-1}$)/dark cycle in a growth chamber (25 °C) for 8 days.

Construction of expression vectors of *Arabidopsis* DWF4 and expression of recombinant DWF4 protein in *E. coli*

The procedure used to assay triazole binding was as described previously [15]. *E. coli* JM109 cells were transformed with the pCW–DWF4 construct. A 10 ml overnight culture of the transformed cells in Luria–Bertani medium supplemented with 100 μg/ml ampicillin was used to inoculate 1 litre of modified Terrific Broth medium supplemented with 100 μg/ml ampicillin, 0.2 % (w/v) glucose and 0.5 mM δ-aminolaevulinic acid. The medium was placed in a 2 litre culture flask and shaken at 37 °C and 225 rev./min. After 3 h, isopropyl β-D-thiogalactoside (0.1 mM) was added to the culture to induce expression of the recombinant protein. The culture was continuously shaken at 30 °C and 150 rev./min for 48 h. Cells from a 1 litre culture were pelleted and resuspended in 50 ml of buffer A, composed of 20 mM potassium phosphate, pH 7.25, 20 % (v/v) glycerol, 1 mM EDTA, 0.1 mM dithiothreitol and 1 mM PMSF. The suspension was sonicated 15 times for 30 s, and bacterial membranes were prepared by centrifugation at 25000 ***g*** for 1 h at 4 °C. The membrane was resuspended in the same buffer supplemented with 1 % (v/v) Triton X-100 (buffer B), and was stirred at 4 °C for 1 h. The solubilized supernatant was collected by centrifugation at 25000 ***g*** for 1 h at 4 °C. The fraction was applied to a Q Sepharose column equilibrated with buffer B. The recombinant DWF4 protein was eluted by linearly increasing the concentration of NaCl in buffer B from 20 to 500 mM. The P450-enriched fractions were used for spectral analysis of the recombinant DWF4 protein.

Assaying triadimefon binding to the recombinant DWF4 protein

The P450 content was determined spectrophotometrically using the molar absorption coefficient difference of the reduced CO difference spectrum ($\Delta\epsilon$ 91.1 $mM^{-1} \cdot cm^{-1}$). The binding of triadimefon compounds to DWF4 protein was determined by measuring the triazole-induced spectral change of the oxidized DWF4 protein. The triadimefon compounds dissolved in DMSO were added to the recombinant DWF4 protein (200 pmol/ml) in buffer B at inhibitor concentrations from 0.1 to 100 μM (final DMSO concentration > 3 %). The difference spectra for each titration set were generated by successive subtraction of the spectrum of the inhibitor-free enzyme from the spectrum after each addition of inhibitor. All spectra were recorded using a Shimadzu UV3100 spectrophotometer.

Seedling treatment and cDNA preparation

Arabidopsis thaliana (ecotype Columbia) seedlings were grown on half-strength Murashige and Skoog medium [26] containing 1.5 % (w/v) sucrose and 0.8 % (w/v) agar for 7 days in light supplemented with or without triadimefon at a final concentration of 10^{-5} M. Cultured seedlings were collected and frozen in liquid nitrogen and stored at −70 °C before RNA extraction. Total RNA was extracted by the guanidinium chloride method [27] and treated with DNase I (Takara Shuzo, Kyoto, Japan). It was then used to synthesize cDNA using the SuperScript™ first-strand synthesis system for reverse transcriptase (RT)-PCR (Invitrogen) according to the manufacturer's instructions.

Quantitative PCR analysis

Quantitative RT-PCR was performed using real-time monitoring TaqMan technology [28] with a Model 7700 sequence detector (Applied Biosystems) according to the manufacturer's instructions. Gene-specific primers, CPD-SP primer (CCCAAACCACTTCAAAGATGCT) and CPD-AP primer (GGGCCTGTCGTTACCGAGTT), and a TaqMan probe CPD-AT (TCTGCCATCTCCAAGGGTTGAAAGTGC) were used to quantify transcripts of the *CPD* gene. Serial dilutions of a full-length cDNA clone of the *CPD* gene, 63C12 (GenBank/EMBL/DDBJ accession number T41675), were used as a standard to quantify the DNA concentration. The 18 S rRNA transcription was analysed with gene-specific primers (forward primer: CGGCTACCACATCCAAGGAA; reverse primer: GCTGGAATTACCGCGGCT) and a TaqMan probe (TGCTGGCACCAGACTTGCCCTC) as an internal control to monitor the efficiency of the RT-PCR reaction. Transcript abundance of the 18 S rRNA was used to normalize the CPD transcript abundance in each sample.

RESULTS

Effect of triadimefon on both dark- and light-grown *Arabidopsis*

Arabidopsis mutants, such as *det2* [29,30] and *cpd* [4], show strong dwarfism with curly dark green leaves when grown in the light, and a de-etiolated phenotype with short hypocotyls and

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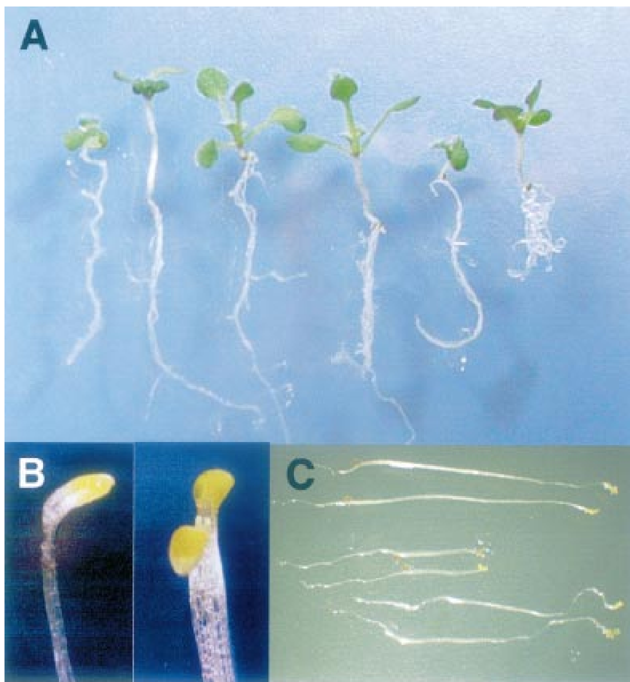

Figure 2 Phenotypes of triadimefon-treated *Arabidopsis*

(**A**) Triadimefon-treated *Arabidopsis* (10-day-old grown in the light) shows a BR-deficient mutant (*det2*)-like phenotype, which is rescued by the application of BL (10 nM). This panel shows (from left to right): *det2*, BL-treated *det2*, wild-type, BL-treated wild-type, wild-type grown on 2 μM triadimefon, and BL-treated wild-type grown with 2 μM triadimefon. (**B**) Triadimefon-treated *Arabidopsis* (10-day-old grown in the dark) shows the BR-deficient phenotype. This panel shows (from left to right): wild-type, wild-type grown on 2 μM triadimefon. (**C**) Triadimefon-treated *Arabidopsis* (10-day-old grown in the dark) shows the BR-deficient phenotype, which is rescued by the application of BL (10 nM). This panel shows (from top to bottom, two seedlings/sample): wild-type, wild-type grown on 2 μM triadimefon, BL-treated wild-type grown on 2 μM triadimefon.

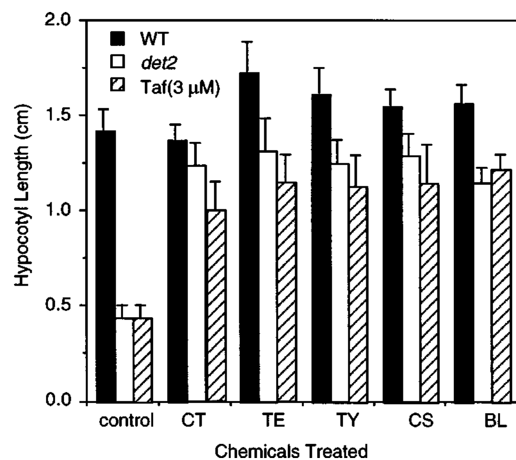

Figure 3 Triadimefon-treated *Arabidopsis* (10-day-old) hypocotyl elongation in the dark in response to applied cathasterone, teasterone, typhasterol, castasterone and BL

Data are the means ± S.E.M. obtained from 30 seedlings; black bars represent the data for the wild-type (WT), white bars represent the data for *det2*, and bars shaded with diagonal lines represent the data for wild-type grown on 3 μM triadimefon (TAf). The concentrations of BR-biosynthesis intermediates and abbreviations are as follows: cathasterone (CT, 1 μM); teasterone (TE, 1 μM); typhasterol (TY, 1 μM); castasterone (CS, 100 nM); and BL (10 nM).

open cotyledons, which are characteristic of light-grown plants, when grown in the dark. This phenotype is rescued by the application of BL, but other plant hormones, such as auxin and GA, have no effect [2]. Consequently, we tested triadimefon in an *Arabidopsis* seedling assay. In the light, triadimefon caused marked malformation of seedlings, which appeared morphologically similar to BR-deficient mutants, such as *det2* (Figure 2A). These triadimefon-induced phenotypes were rescued by the co-application of 10 nM BL. In the dark, triadimefon induced a de-etiolated phenotype with open cotyledons (Figure 2B) and a short hypocotyl, similar to that of *det2*. These phenotypes were also rescued by the application of 10 nM BL (Figure 2C). In the reversion test, plants were sensitive to the growth conditions, perhaps because of slow BL uptake and transport within *Arabidopsis*. This might be one of the reasons why BRs did not completely reverse the inhibition by triadimefon and the *det2* mutant in our assays in the dark. It is also possible that triadimefon has a side effect that cannot be overcome by the application of BL. This possible side effect of triadimefon will be discussed below.

Feeding BR-biosynthesis intermediates to triadimefon-treated *Arabidopsis*

To investigate the biosynthetic step(s) affected by triadimefon, we examined the effect of biosynthetic intermediates downstream from cathasterone on hypocotyl elongation of triadimefon-treated *Arabidopsis* and *det2* seedlings according to a method reported previously [8]. Non-treated seedlings were not affected by 1 μM cathasterone and teasterone, 100 nM castasterone, or 10 nM BL. However, these concentrations of teasterone, castasterone and BL rescued both triadimefon-treated and *det2* hypocotyl growth (Figure 3). Cathasterone was less effective in rescuing the triadimefon-treated hypocotyl growth, but was effective in rescuing *det2* hypocotyl growth, as reported previously [30]. This result was the same as that obtained in brassinazole-treated *Arabidopsis*.

Binding assay of triadimefon to DWF4 protein

Brassinazole binds to DWF4 [15] and does not bind to CPD (M. Mizutani, unpublished work), and this binding to DWF4 was ascribed as the reason for the potency of brassinazole as a BR-biosynthesis inhibitor. Since triadimefon was suggested to have the same target site as brassinazole, we analysed the interaction between DWF4 expressed in *E. coli* and triadimefon in order to investigate whether DWF4 is a target of triadimefon. The dissociation constant (K_d) was determined using the method described in our previous study [12] (Figure 4). The K_d for triadimefon was determined as 2.5×10^{-6} M. The dissociation constant for brassinazole (1.0×10^{-6} M) was lower than that for triadimefon, demonstrating a greater affinity. Figure 5 shows the activity of brassinazole and triadimefon in inhibiting hypocotyl growth of *Arabidopsis* grown in the dark. The results indicated that the binding affinity and activity in inhibiting hypocotyl growth are probably correlated: triadimefon had a lower affinity and a lower inhibitory activity, and the concentration required for 50 % inhibition of hypocotyl growth was about three times higher than the required concentration of brassinazole.

Feedback regulation of CPD mRNA accumulation by triadimefon treatment

Previous analysis of endogenous BRs in several BR-biosynthesis dwarf mutants revealed that active BRs are deficient in these

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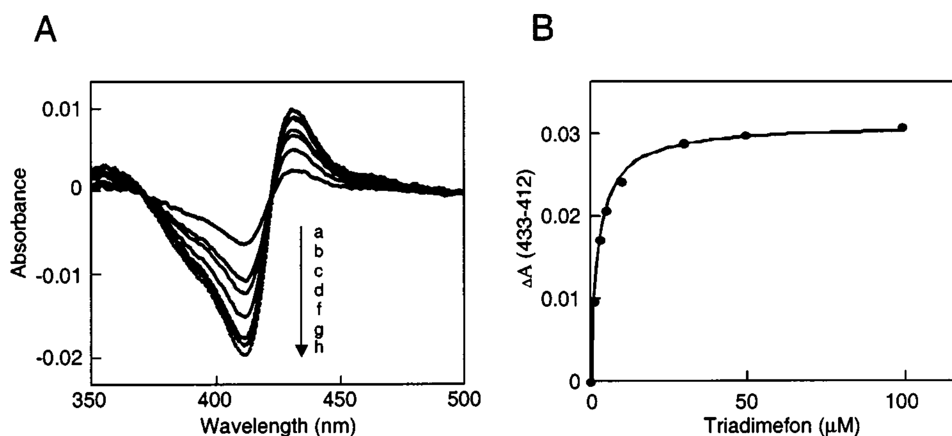

Figure 4 Binding of triadimefon to DWF4 protein

The recombinant DWF4 protein (200 pmol/ml) was dissolved in 50 mM potassium phosphate buffer, pH 7.25, containing 20% (v/v) glycerol and 0.5% (w/v) Triton X-100. (**A**) Spectrophotometric titration of DWF4 with triadimefon. Triadimefon was added to the DWF4 protein at different final concentrations (1, 3, 5, 10, 30, 50 and 100 μM for traces a, b, c, d, f, g and h respectively), and triadimefon-induced difference spectra were recorded. (**B**) A plot of absorbance differences ΔA (433 − 412 nm) against triadimefon concentrations (μM).

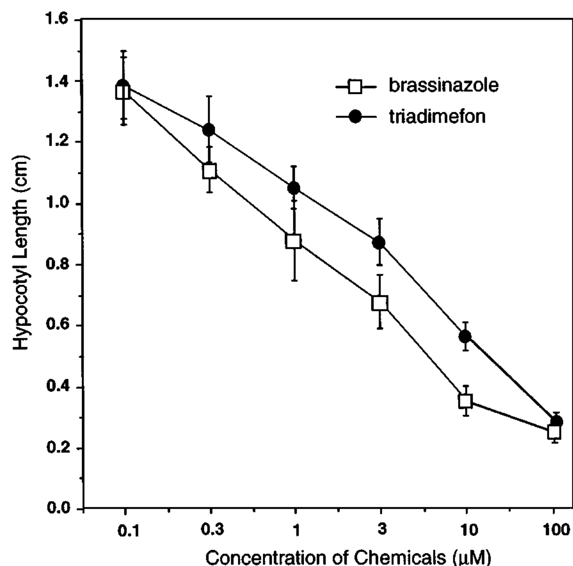

Figure 5 Hypocotyl lengths of dark-grown 10-day-old seedlings of *Arabidopsis* treated with concentrations of brassinazole and triadimefon from 0.1 to 10 μM

Data are the means ± S.E.M. obtained from 30 seedlings.

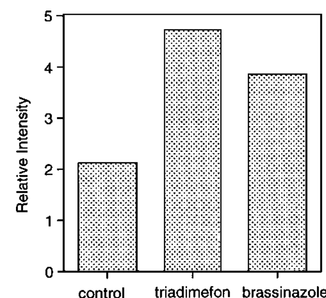

Figure 6 Up-regulation of *CPD* gene expression by brassinazole or triadimefon treatment

Arabidopsis seedlings were grown in half-strength Murashige and Skoog agar medium for 7 days in the presence or absence of 1 μM brassinazole or triadimefon in the light. Transcript abundance was analysed using the TaqMan real-time quantitative RT-PCR. Transcript abundance levels are given as relative values normalized to 18 S ribosomal RNA levels.

mutants. BR-insensitive dwarf mutants of *Arabidopsis* (*bri1-4*) accumulated very high levels of BL, castasterone and typhasterol, indicating that the *BRI1* gene is required for feedback regulation of BR biosynthesis [31]. Treatment of *Arabidopsis* with specific BR intermediates or end products, such as BL or castasterone, reduced the CPD mRNA levels [32,33]. By contrast, Noguchi et al. [34] reported the up-regulation of genes committed to BR biosynthesis at the transcription level due to BR deficiency. On the basis of these facts, we examined the CPD mRNA levels in *Arabidopsis* seedlings grown for 7 days in the presence of triadimefon using real-time quantitative RT-PCR. The *CPD* gene was up-regulated in both triadimefon-treated *Arabidopsis* seedlings and brassinazole-treated seedlings (Figure 6).

Triadimefon affects both BR and GA biosynthesis

To compare the effects of brassinazole, triadimefon and uniconazole (a GA-biosynthesis inhibitor) on plant growth, these compounds were applied to cress, because we have previously demonstrated that cress is very sensitive to internal BR or GA deficiency, and it is a useful plant for evaluating BR- or GA-biosynthesis inhibitors [8].

Triadimefon-treated cress developed a phenotype similar to that of brassinazole-treated cress, with curly dark-green leaves in the light. The hypocotyl length of cress seedlings treated with 0.1 μM uniconazole, 1 μM brassinazole or 1 μM triadimefon was 21, 25 and 23 % of the control respectively (Figure 7). Cress treated with BL showed approx. 125 % elongation, possibly due to the intrinsic effect of BL on growth stimulation. Cress treated with both 0.1 μM uniconazole and 10 nM BL showed 27 % elongation, whereas cress treated with 1 μM brassinazole and 10 nM BL or 1 μM triadimefon and 10 nM BL showed 105 or

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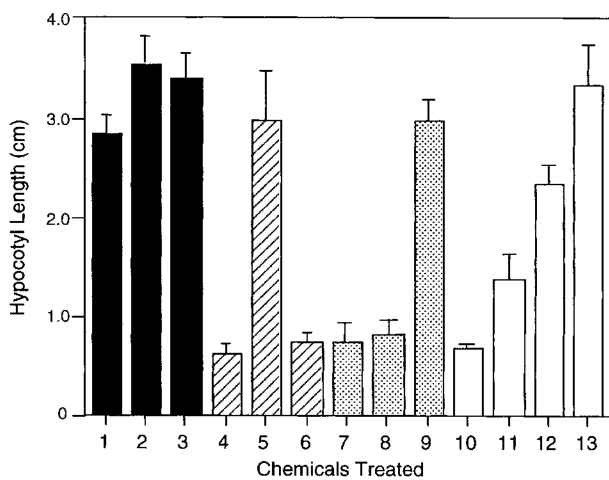

Figure 7 Average hypocotyl length of cress grown on medium including a variety of chemicals

Each bar is indicated by a number, representing the following experimental set-up: 1, control; 2, BL (10 nM); 3, GA_3 (1 μM); 4, uniconazole (Uni; 0.1 μM); 5, Uni (0.1 μM) + GA_3 (1 μM); 6, Uni (0.1 μM) + BL (10 nM); 7, brassinazole (Brz; 1 μM); 8, Brz (1 μM) + GA_3 (1 μM); 9, Brz (1 μM) + BL (10 nM); 10, triadimefon (Taf; 1 μM); 11, Taf (1 μM) + GA_3 (1 μM); 12, Taf (1 μM) + BL (10 nM); and 13, Taf (1 μM) + GA_3 (1 μM) + BL (10 nM). Data are the means ± S.E.M. obtained from 20 seedlings.

82 % elongation respectively. Dwarfism induced by triadimefon treatment was reduced by the co-application of BL. Cress treated with GA_3 showed approx. 118 % elongation, which could be due to the intrinsic effect of GAs on growth stimulation. Cress treated with both 0.1 μM uniconazole and 1 μM GA_3 showed 105 % elongation, whereas cress treated with 1 μM brassinazole and 1 μM GA_3 or 1 μM triadimefon and 1 μM GA_3 showed approx. 28 or 48 % elongation respectively. Taken together, these results show that GA_3 rescued brassinazole-treated cress only minimally, but triadimefon-treated cress fairly well (Figure 7). When cress was treated with uniconazole, it responded well to GA treatment; when cress was treated with brassinazole, it responded well to BL treatment; and triadimefon-treated cress responded well to both BL and GA. Cress treated with the combination of triadimefon, BL and GA showed almost no dwarfism.

DISCUSSION

BRs are distributed widely throughout the Plant Kingdom, along with biosynthetically related compounds. One of the advantages of using BR-biosynthesis inhibitors for analysing the roles of BRs in plants, rather than other BR-deficient mutants, is that they can be applied to a variety of plant species. In our experiments, triadimefon-treated dicotyledonous plants showed morphological changes similar to those of BR-deficient mutants of *Arabidopsis*: strong dwarfism with curly, dark-green leaves in the light, and a de-etiolated phenotype with short hypocotyls and open cotyledons [8]. This suggests that triadimefon affects BR biosynthesis, perhaps by targeting cytochrome P450s in the BR-biosynthesis pathway, since it includes a triazole ring, which is widely accepted as binding to the iron atom in the haem of cytochrome P450s. $BR6_{ox}$ [35], DDWF1 [7], CPD, DWF4 and 14DM are cytochrome P450s that act in the *Arabidopsis* BR-biosynthesis pathway. BRs cannot rescue the phenotype of antisense 14DM plants [36] or the *fackel* mutant [37,38] in which sterol C-14 reductase is mutated. Sterol C-14 reductase acts one step downstream from 14DM in BR biosynthesis. These results suggest that 14DM is not the target of triadimefon. Combined with the results of the feeding experiment, this suggests that the target of triadimefon could be the two-step conversion of 6-oxocampesterol into cathasterone to teasterone catalysed by DWF4 and CPD respectively. Considering the similar functions and amino acid sequences of DWF4 and CPD, it is reasonable to speculate that triadimefon inhibits the steps catalysed by both enzymes. Triadimefon bound to DWF4, but not to CPD, when both were expressed in *E. coli*. The binding constant of triadimefon was about 2.5 times larger than that of brassinazole, and this probably corresponds to the activity difference between the two inhibitors in the *in planta Arabidopsis* hypocotyl growth test. That is, triadimefon requires a concentration between two and three times greater than brassinazole for 50 % inhibition of hypocotyl growth. Therefore we conclude that the main target site of triadimefon is DWF4.

Until now, except for brassinosteroid-6-oxidase in tomato and *Arabidopsis*, none of the activities of the plant P450s involved in BR hormone biosynthesis have been confirmed by functional assays. In our *in vitro* assay system, campestanol, the putative substrate of DWF4, was applied to *E. coli* cells expressing recombinant DWF4 protein. However, we could not detect any hydroxylated product after incubation for 24 h at 30 °C. This is probably due to the lack of an electron-transport system (i.e. NADPH-cytochrome P450 reductase, cytochrome b_5, NADH–cytochrome b_5 reductase) in *E. coli* cells. Therefore, although triadimefon had a high binding affinity for the DWF4 protein, we could not confirm the direct inhibition of the hydroxylation activity of the DWF4 protein by triadimefon. In the future, the target sites of triadimefon should be identified directly in functional assays of DWF4 and CPD.

A reduction in BR signals increases the level of mRNA encoding biosynthetic enzymes by feedback regulation [15,32,33]. The feedback regulatory factor was suggested to be translated *de novo*, since regulation was abolished by the presence of the protein synthesis inhibitor, cycloheximide [32]. In this context, we examined the CPD mRNA levels in *Arabidopsis* 7 days after treatment with triadimefon (Figure 6). The CPD mRNA level in triadimefon-treated *Arabidopsis* clearly increased within 3 h. This rise in mRNA level suggests that the messenger is turned over rapidly.

In summary, we obtained three pieces of evidence that triadimefon treatment of plants induces BR deficiency. (1) The *Arabidopsis* phenotype induced by triadimefon treatment is like that of BR-deficient mutants, and this phenotype is reversed to the non-treated phenotype by the co-application of BL and triadimefon. (2) Triadimefon shows good binding affinity to expressed DWF4 protein, and this affinity is probably correlated with its *in planta* activity. (3) Triadimefon treatment induces CPD mRNA accumulation, which was shown in both BR-deficient mutants and brassinazole-treated plants. Moreover, the results shown in Figure 7 suggest that triadimefon targets both the BR- and GA-biosynthesis pathways. These results explain the phenotypic changes seen in plants treated with triadimefon. Since triadimefon is beneficial to plant growth, chemical modification of triadimefon should lead to the discovery of new plant growth regulators.

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